Human Papillomavirus 16 E6 Antibodies in Individuals Without Diagnosed Cancer: A Pooled Analysis

Running Title: HPV16 E6 Antibodies Without Cancer

Krystle A. Lang Kuhs[1*], Devasena Anantharaman[2*], Tim Waterboer[3], Mattias Johansson[2], Paul Brennan[2], Angelika Michel[3], Martina Willhauck-Fleckenstein[3], Mark P. Purdue[1], Ivana Holcátová[4], Wolfgang Ahrens[5], Pagona Lagiou[6], Jerry Polesel[7], Lorenzo Simonato[8], Franco Merletti[9], Claire M. Healy[10], Kristina Kjaerheim[11], David I. Conway[12], Tatiana V. Macfarlane[13], Peter Thomson[14], Xavier Castellsagué[15], Ariana Znaor[16], Amanda Black[1], Wen-Yi Huang[1], Vittorio Krogh[17], Antonia Trichopoulou[18,19,20], H.B(as). Bueno-de-Mesquita[21,22,23,24], Françoise Clavel-Chapelon[25,26,27], Elisabete Weiderpass[28,29,30,31], Johanna Ekström[32], Elio Riboli[33], Anne Tjønneland[34], María-José Sánchez[35,36], Ruth Travis[37], Allan Hildesheim[1], Michael Pawlita[3] and Aimée R. Kreimer[1]

*Indicates co-first authors

Affiliations

[1]National Cancer Institute, NIH, Bethesda, Maryland, USA

[2]International Agency for Research on Cancer (IARC), Lyon, France

[3]German Cancer Research Center (DKFZ), Heidelberg, Germany

[4]Charles University in Prague, Prague, Czech Republic

[5]University Bremen, Bremen, Germany

[6]University of Athens Medical School, Athens, Greece

[7]CRO Aviano National Cancer Institute, Aviano, Italy

[8]University of Padova, Padova, Italy

[9]CeRMS and University of Turin, Turin, Italy

[10]Trinity College School of Dental Science, Dublin, Ireland

[11]Cancer Registry of Norway, Oslo, Norway

[12]University of Glasgow, Glasgow, United Kingdom; and NHS NSS, Edinburgh, UK

[13]University of Aberdeen, Aberdeen, United Kingdom

[14]University of Newcastle on Tyne, Newcastle, United Kingdom

[15]Unit of Infections and Cancer, Institut Català d'Oncologia (ICO), IDIBELL, CIBERESP, L'Hospitalet de Llobregat, Catalonia, Spain

[16]Croatian National Institute of Public Health, Zagreb, Croatia

[17]Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

[18]Hellenic Health Foundation, Athens, Greece

[19]Academy of Athens, Athens, Greece

[20]University of Athens Medical School, Athens, Greece

[21]National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands

[22]University Medical Centre, Utrecht, The Netherlands

[23]The School of Public Health, Imperial College London, London, United Kingdom

[24]University of Malaya, Kuala Lumpur, Malaysia

[25]Centre for Research in Epidemiology and Population Health (CESP), Villejuif, France

[26]Université Paris Sud, Villejuif, France

[27]Institut Gustave Roussy, Villejuif, France

[28]University of Tromsø, The Arctic University of Norway, Tromsø, Norway

[29]Cancer Registry of Norway, Oslo, Norway

[30]Karolinska Institutet, Stockholm, Sweden

[31] Folkhälsan Research Center, Helsinki, Finland

[32]Lund University, Lund, Sweden

[33]Imperial College London, London, United Kingdom.

[34]Danish Cancer Society Research Center, Copenhagen, Denmark

[35]Hospitales Universitarios de Granada/Universidad de Granada, Granada, Spain

[36]CIBER de Epidemiología y Salud Pública (CIBERESP), Madrid, Spain

[37]University of Oxford, Oxford, United Kingdom

Correspondence to:

Krystle A. Lang Kuhs, Ph.D., MPH

Infections and Immunoepidemiology Branch

Division of Cancer Epidemiology and Genetics, National Cancer Institute

9609 Medical Center Drive, RM 6-E210

Bethesda, MD 20892

(240) 276-7177

Krystle.Kuhs@nih.gov

ABSTRACT

BACKGROUND: The increasing incidence of oropharyngeal cancer in many developed countries has been attributed human papillomavirus type 16 (HPV16) infections. Recently, HPV16 E6 serology has been identified as a promising early marker for oropharyngeal cancer. Therefore, characterization of HPV16 E6 seropositivity among individuals without cancer is warranted. METHODS: 4,666 controls were pooled from several studies of cancer and HPV seropositivity, all tested within the same laboratory. HPV16 E6 seropositive controls were classified as having i) moderate seroreactivity (MFI ≥484 & <1000) or ii) high seroreactivity (MFI ≥ 1000). Associations of moderate and high HPV16 E6 seroreactivity with i) demographic risk factors; and seropositivity for ii) other HPV16 proteins (E1, E2, E4, E7 and L1) and iii) E6 proteins from non-HPV16 types (HPV6, 11, 18, 31, 33, 45 and 52) were evaluated. RESULTS: Thirty-two (0.7%) HPV16 E6 seropositive controls were identified; 17 (0.4%) with moderate and 15 (0.3%) with high seroreactivity. High HPV16 E6 seroreactivity was associated with former smoking (OR 5.5 [95%CI: 1.2-51.8]), and seropositivity against HPV16 L1 (OR 4.8, 95%CI: 1.3-15.4); E2 (OR 7.7, 95%CI: 1.4-29.1); multiple HPV16 proteins (OR 25.3, 95%CI: 2.6-119.6 for 3 HPV16 proteins beside E6) and HPV33 E6 (OR 17.7, 95%CI: 1.9-81.8). No associations were observed for controls with moderate HPV16 E6 seroreactivity. CONCLUSIONS: High HPV16 E6 seroreactivity is rare among individuals without diagnosed cancer and was not explained by demographic factors. Some HPV16 E6 seropositive individuals, especially those with seropositivity against other HPV16 proteins, may be on the path to an HPV-driven cancer.

INTRODUCTION

A rapid increase in the incidence of oropharyngeal cancer has been reported in many parts of the world with a high development index (1-8), especially among men younger than 60 years of age (9). This upsurge has been attributed to an increase in HPV-driven oropharyngeal cancers (7). In the US, incidence has increased by more than 200 percent over the past several decades (10). HPV16 infection alone accounts for approximately 90 percent of HPV-positive oropharyngeal cancers (11, 12) and is estimated to be responsible for at least 50% of oropharyngeal cancer cases in parts of the world with a high development index (10, 13, 14).

Unlike cervical cancer, a precursor lesion for oropharyngeal cancer has yet to be identified, making early detection of oropharyngeal cancers difficult (15). However, numerous case-control studies have shown that the presence of circulating HPV antibodies is strongly associated with cancer of the oropharynx (12, 16-24). Recently, HPV16 E6 antibody positivity has been identified as a potentially promising marker for oropharyngeal cancer (25). A prospective study conducted with prediagnostic sera found that 35% of patients with oropharyngeal cancer were seropositive for HPV16 E6 compared to only 0.6% of controls; for some of the patients these antibodies were present more than 10 years prior to diagnosis and were not associated with cancers at other head and neck cancer sites (25).

Due to the high specificity of HPV16 E6 seropositivity for oropharyngeal cancer, this marker has the potential to be further developed into a screening tool for identifying high-risk individuals. Therefore, characterization of HPV16 E6 seropositivity within healthy individuals without diagnosed cancer is merited. However, HPV16 E6 seropositivity is extremely rare among healthy individuals without cancer (<1%), making it difficult to adequately study (23-25).

To overcome this issue, we conducted a descriptive epidemiological analysis of pooled controls from several studies of cancer and HPV seropositivity whose samples were all tested within the same laboratory with a bridging panel that allowed for interpretation across studies (23-25). The goals of this analysis were to investigate demographic and serologic factors associated with HPV16 E6 seropositivity among individuals without diagnosed cancer.

METHODS

Our analytic population consisted of 4,666 controls pooled from several large studies of HPV seropositivity; 3 studies of head and neck cancer and 1 study of anogenital cancers (23-26). Controls were pooled from i) two nested case-control investigations within the European Prospective Investigation Into Cancer and Nutrition (EPIC); one focused on head and neck cancer (n=1,599 controls) and one focused on HPV-driven anogenital cancers (n=718 controls) (25, 26); ii) 1 nested case-control investigation within the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO) (n=924 controls) (27); and iii) 1 case-control study, the Alcohol-Related Cancers and Genetic Susceptibility in Europe (ARCAGE) (n=1,425 controls) (23).

Description of Study Populations and Participant Selection

EPIC Cohort Study

In brief, 521,330 individuals were recruited to the cohort between 1992 and 2000 from 10 European countries, of whom 385,747 participants contributed a blood sample (28). Participants completed self-administered questionnaires on lifestyle factors and diet. Two control participants (one in Denmark) were randomly assigned for each patient with cancer (including head and neck and anogenital cancers) from appropriate risk sets consisting of all cohort participants alive and free of cancer (except non-melanoma skin cancer) at the time of diagnosis (and hence, age) of the index case. Additional study specific matching criteria are discussed below.

EPIC Head and Neck Study

Matching criteria were: country, sex, date of blood collection (± 1 month, relaxed to ± 5 months for sets without available controls), and date of birth (±1 year, relaxed to ± 5 years for sets without available participants). The final study included 1,599 controls (25).

EPIC Anogenital Study

Matching criteria included: study center, sex, date of blood collection (± 3 months, relaxed to ± 6 months for sets without available controls), and age at blood collection (± 3 months, relaxed to ± 2 years for sets without available controls), fasting status, and where relevant, menopausal status, and postmenopausal hormone replacement therapy use, and menstrual cycle. The final study included 718 controls (26).

PLCO Cohort Study

PLCO recruited approximately 155,000 55-74 year-olds from the general population during 1993-2001 who had not been diagnosed previously with prostate, lung, colorectal, or ovarian cancer. Blood (screening arm only), demographic and behavioral data were collected (29). Blood samples were obtained at baseline and five subsequent annual visits; the earliest available sample was used for this study. For each case, four controls were randomly chosen from appropriate risk sets consisting of all cohort members alive and free of cancer (except non-melanoma skin cancer) at the time (and hence age) of diagnosis of the index head and neck cancer case. Matching criteria were: year of entry into the study, year the material was collected, study year of cancer diagnosis (for cases; the same year was used for the matched control), birth year, and smoking status (never, former, current) (26).

ARCAGE Case-Control Study

Briefly, 2,227 control subjects were recruited from 10 European countries during the period from 2002 to 2005 using a standardized protocol in all centers (except France) (30). All subjects underwent personal interviews to record lifestyle exposures; details are described elsewhere (30). Controls were recruited in each center and frequency matched for age, sex, and area of residence to cases with head and neck cancer. ARCAGE centers mainly used hospital controls to facilitate collection of blood samples, with the exception of the UK centers which used population-based controls randomly chosen from the same family medical practice list as the corresponding cases. Hospital controls were selected from the following diagnoses: endocrine and metabolic, skin, subcutaneous tissue and musculoskeletal, circulatory, nervous system diseases, genitourinary, gastro-intestinal, ear, eye and mastoid, plastic surgery cases and trauma patients (23).

Harmonization of Covariates

Given the variation between studies in how the covariates were ascertained, we were unable to create single unified definitions of smoking and alcohol consumption. As a result, individuals were categorized according to study-specific definitions of smoking and alcohol consumption (Supplemental Table 1).

Harmonization of Serologic Test Results

Plasma (EPIC and ARCAGE) and serum (PLCO) samples were sent on dry ice to the German Cancer Research Center (DKFZ, Heidelberg, Germany) and testing was performed using multiplex assays (24, 31-33). Samples were analyzed for HPV16 antibodies to the major capsid protein (L1), the early oncoproteins (E6, E7), and other early proteins (E1, E2, E4) with the exception of PLCO where seroreactivity against HPV16 E4 was not assessed. Additionally, seroreactivity against the E6 protein from the following HPV types was also evaluated; HPV6, HPV11, HPV18, HPV31, HPV33, HPV45 and HPV52 with the exception of the EPIC anogenital study were seroreactivity against HPV52 was not assessed. A bridging panel was included in all studies so that MFI values could be normalized to account for variations in the assay between studies (34). This bridging panel allowed us to apply the same standard MFI cutoff values for seropositivity across all three studies. For HPV16 E6, two mutually exclusive categories of HPV16 E6 seroreactivity were created: i) moderate: MFI $\geq$ 484 and < 1000; and high: MFI $\geq$ 1,000. Previous work from our group showed that increasing the seropositivity cutoff of HPV16 E6 from 484 to 1000 resulted in an increased specificity for oropharyngeal cancer without an associated decrease in sensitivity (25). For the other HPV16 proteins the MFI cutoffs used to define seropositivity were: L1, 422; E1, 200; E2, 679; E4, 876; E6, 484; E7, 548. For the E6 proteins of non-HPV16 types the MFI cutoffs for seropositivity were: HPV6 E6, 500; HPV11, 260; HPV18, 243; HPV31, 890; HPV33, 253; HPV35, 260; HPV45, 249; HPV52, 271.

Statistical Analyses

Characteristics of the control participants were evaluated overall and by study. The proportion of HPV16 E6 seropositive controls by demographic categories was computed for the pooled studies. Demographic and serologic determinants of moderate and high HPV16 E6 seroreactivity were evaluated through odds ratios (OR) and 95% confidence intervals (CI) calculated in univariate analyses by logistic regression. Demographic variables evaluated included: gender, age, world region, smoking status and alcohol consumption. Serologic variables evaluated included seroreactivity against: i) other HPV16 proteins (L1, E1, E2, E4, E7) and ii) E6 proteins from non-HPV16 types (HPV6, HPV11, HPV18, HPV31, HPV33, HPV45 and HPV52).

RESULTS

Participant Characteristics

A total of 4,666 controls without diagnosed cancer were included in this analysis (Table 1); 1425 (30.5%) individuals from ARCAGE; 2,317 (49.7%) from EPIC and 924 (19.8%) from PLCO. The majority of the controls were male (63.7%), 60 years of age or younger (59.6%) and ever alcohol drinkers (80.3%); smoking status appeared evenly distributed among the categories (i.e.: never, former, current). Small differences between studies were noted for gender, age and smoking status (Table 1).

Demographic Determinants of HPV16 E6 Seropositivity

HPV16 E6 seropositivity was rare. Of the 4,666 pooled controls, a total of 32 individuals (0.7%) were seropositive for HPV16 E6. Prevalence of HPV16 E6 seropositivity was similar by study; ARCAGE (0.8%), EPIC (0.6%) and PLCO (0.9%). Of the 32 HPV16 E6 seropositive controls, 17 (0.4%) were classified as having moderate HPV16 E6 seropositivity (MFI $\geq$ 484 and < 1000) and 15 (0.3%) were classified as having high HPV16 E6 seroreactivity (MFI $\geq$ 1000) (Table 2).

Age, gender, smoking status and alcohol consumption did not elevate the odds of moderate or high HPV16 E6 seroreactivity. Only former smoking was significantly associated with high HPV16 E6 seroreactivity, OR 5.5 (95% CI: 1.2-51.8). No other significant associations for either moderate or high HPV16 E6 seroreactivity were observed (Table 2).

Serologic Determinants of HPV16 E6 Seropositivity

Seroreactivity against HPV16 proteins, including L1, E1, E2, E4 or E7, was common. Similar seroprevalence for any of these proteins was observed among the HPV16 E6 seronegative and moderately HPV16 E6 seroreactive controls; 27.9% and 29.4%, respectively (Table 3). No significant associations between moderate HPV16 E6 seroreactivity and seroreactivity against any of the other HPV16 proteins either individually or combined was observed.

In contrast, prevalence of seroreactivity against 1 or more HPV16 proteins (L1, E1, E2, E4 or E7) in addition to E6 was greatest among the controls with high HPV16 E6 seroreactivity; 46.7%. Of the 5 HPV16 proteins evaluated, seroreactivity against HPV16 L1 was most common (n=5 out of 15, 33.3%). Controls with high HPV16 E6 seroreactivity were also more likely than HPV16 E6 seronegative controls to be seroreactive against all HPV16 proteins with the exception of E7, however only HPV16 L1 (OR 4.3, 95% CI: 1.1-13.8) and E2 (OR 7.7, 95% CI: 1.4-29.1) reached statistical significance. High HPV16 E6 seroreactivity was also significantly associated with seroreactivity against multiple HPV16 proteins; OR 25.3 (95% CI: 2.6-119.6) for seroreactivity against 3 HPV16 proteins in addition to E6, although in absolute terms, seroreactivity against multiple HPV16 proteins was rare (4 of 4666; 0.08%).

Seroreactivity against E6 proteins from non-HPV16 types was less common; 7.9% of HPV16 E6 seronegative controls were seroreactive against any non-HPV16 type compared to approximately 20% among both moderate and high HPV16 E6 seroreactive controls. Only seroreactivity for HPV33 E6 was significantly associated with high HPV16 E6 seroreactivity; OR 17.7 (95% CI: 1.9-81.8, Table 4).

DISCUSSION

In this large descriptive epidemiological analysis of more than 4,000 pooled controls from several studies of HPV seroreactivity and HPV-associated cancer (23-25), HPV16 E6 seropositive controls were rare (<1%), particularly those with high HPV16 E6 seroreactivity (0.3%). Further, of the determinants of HPV16 seropositivity evaluated, significant associations were observed only among controls with high HPV16 E6 seroreactivity. Of the demographic risk factors assessed, none were predictors of HPV16 E6 seropositivity except for former smoking. Of the serologic determinants assessed, seroreactivity against other HPV16 proteins was common among all controls and was greatest among controls with high HPV16 E6 seroreactivity (47%). A marker of cumulative lifetime HPV16 exposure, HPV16 L1 seropositivity (5 out of 15; 33.3%) was most commonly detected among high HPV16 E6 seroreactive controls compared to the other 4 HPV16 proteins tested. High HPV16 E6 seroreactivity was significantly associated with seroreactivity against HPV16 L1 (OR 4.8, 95% CI: 1.3-15.4), E2 (OR 7.7, 95% CI: 1.4-29.1) and multiple HPV16 proteins (OR 25.3, 95% CI: 2.6-119.6 for seroreactivity against any 3 HPV16 proteins in addition to E6). Seropositivity for any non-HPV16 E6 proteins was less common; 8% among HPV16 E6 seronegative controls and approximately 20% among controls with both moderate and high HPV16 E6 seroreactivity. Only HPV33 E6 seroreactivity was significantly associated with high HPV16 E6 seroreactivity; OR 17.7 (95% CI: 1.9-81.8), however due to its high sequence homology with the E6 protein of HPV16, this is finding may be the result of antibody cross-reactivity (35).

Theoretically, individuals without an underlying HPV-driven cancer would not be expected to have antibodies against the HPV16 oncoproteins. One potential explanation for the small percentage of controls with seroreactivity against HPV16 E6 may be due to potential laboratory error or sample misclassification. Therefore, the 15 strongly HPV16 E6 seroreactive controls identified may reflect the error rates within these large epidemiological studies. Alternatively, the HPV16 E6 seroreactive controls identified in our study may be harboring a yet to be diagnosed cancer or precancer. Recent findings have suggested that induction of HPV-specific antibodies, most notably HPV16 E6 antibodies, may be a response to an underlying HPV-driven neoplastic process that may take years to manifest into a diagnosable cancer (25). Studies conducted at the time of diagnosis have shown that the presence of detectable HPV16-specific antibodies in diagnostic serum is highly sensitive for HPV-driven head and neck squamous cell carcinomas (36). A large proportion (7 out of 15) of controls with high HPV16 E6 seroreactivity was also seroreactive against at least one other HPV16 protein in addition to HPV16 E6. However, of all HPV16 proteins, a recent prospective study showed that seroreactivity against HPV16 E6 is the most strongly associated with development of oropharyngeal cancer; OR 274 (95% CI: 110-681). Of note, all the HPV16 E6 seropositive oropharyngeal cancer cases identified in the previous study had HPV16 E6 MFI values greater than 1,000 and therefore would have been classified as having high seroreactivity in this current analysis (25). Taken together, these findings raise the possibility that the HPV16 E6 seropositive controls described in this study may be on the path to developing an HPV-driven cancer or precancer that, as follow-up accrues over time may eventually be diagnosed. Updated record linkage of the EPIC head and neck cancer study (25) revealed that one HPV16 E6 seropositive control was subsequently diagnosed with invasive anal cancer, however, additional follow-up time is needed to fully investigate this possibility.

Therefore, an important limitation of this study is that we are unable to extend follow-up to further ascertain the health status of the HPV16 E6 seropositive controls. For the cohort studies

(EPIC and PLCO), we will continue to monitor record linkage updates and investigate this question accordingly; for the ARCAGE case-control studies, no additional follow-up will become available. Additionally, controls in this analysis were initially matched to cases, therefore skewing their distribution of certain variables, such as age and gender, towards that of cases. For the case-control studies, controls were recruited only when they were eligible based on a list of non-chronic diseases unrelated to smoking and alcohol. While this does not jeopardize the validity of our findings, it limits their generalizability. There may have also been some minor misclassification in terms of smoking status and alcohol consumption due to the differences between studies in how these variables were ascertained on the questionnaires. Additionally, we do not have information regarding other covariates such as sexual behavior, and host immunogenetics, which may have been potentially helpful in explaining why some individuals develop these antibodies. Finally, even with over 4,000 pooled controls, we were still limited by power given that our outcome was so rare.

Due to the high specificity of the HPV16 E6 marker (25), HPV16 E6 antibody testing may have the potential to be further developed into a screening tool for identifying individuals at high-risk for oropharyngeal cancer. Characterization of HPV16 E6 seropositivity within individuals without diagnosed cancer has important implications in terms of determining the utility of this marker as a screening tool. Future prospective studies should aim to determine the positive predictive value of HPV16 E6 positivity for the diagnosis of oropharyngeal cancer.

Acknowledgements

We would like to thank the following people for their contributions to the manuscript: David Castenson (Information Management Services, Calverton MD, USA); Dana Mates (Institute of Public Health, Bucharest, Romania); Eleonora Fabianova (Specialized Institute of Hygiene and Epidemiology, Banska Bystrica, Slovakia); Vladimir Bencko (Charles University in Prague, Czech Republic); Jolanta Lissowska (Department of Cancer Epidemiology and Prevention Cancer Center and M. Sklodowska-Curie Memorial Institute of Oncology, Warsaw, Poland); Lenka Foretova (Department of Cancer Epidemiology and Genetics, Masaryk Memorial Cancer Institute and MF MU, Brno, Czech Republic); Victor Wünsch-Filho (School of Public Health, University of Sao Paulo, Sao Paulo, Brazil); Elena Matos (Institute of Oncology Angel H. Roffo, University of Buenos Aires, Buenos Aires, Argentina); Jose Eluf-Neto (Universidade de São Paulo, Sao Paulo, Brazil) and Leticia Fernandez (Instituto Nacional de Oncologia de Cuba).

Funding

Palli is supported by a grant from Associazione Italiana per la Ricerca sul Cancro-AIRC-Italy. Ramón Quirós recevies funds from the Regional Government of Asturias. The EPIC study has been supported by the Europe Against Cancer Program of the European Commission (SANCO); Deutsche Krebshilfe, Deutsches Krebsforschungszentrum, German Federal Ministry of Education and Research; Danish Cancer Society; Health Research Fund (FIS) of the Spanish Ministry of Health, Spanish Regional Governments of Andalucia, Asturias, Basque Country, Murcia and Navarra; Catalan Institute of Oncology, Spain; the ISCIII of the Spanish Ministry of Health (RETICC DR06/0020); Cancer Research UK; Medical Research Council, United Kingdom; Greek Ministry of Health; Stavros Niarchos Foundation; Hellenic Health Foundation;

Italian Association for Research on Cancer (AIRC); Italian National Research Council, Fondazione-Istituto Banco Napoli, Italy; Associazione Italiana per la Ricerca sul Cancro-AIRC-Milan; Compagnia di San Paolo; Dutch Ministry of Public Health, Welfare and Sports; World Cancer Research Fund; Swedish Cancer Society; Swedish Scientific Council; Regional Government of Västerbotten, Sweden; NordForsk (Centre of excellence programme HELGA), Norway; French League against Cancer (LNCC), France; National Institute for Health and Medical Research (INSERM), France; Mutuelle Générale de l'Education Nationale (MGEN), France; 3M Co, France; Gustave Roussy Institute (IGR), France; and General Councils of France.

The ARCAGE study was supported by the grant from European Commission's 5th Framework Program (contract QLK1-2001-00182). This project was partly funded by the Health General Directorate of the French Social Affairs and Health Ministry. The serology testing was supported in part by a grant from the European Commission's 7th Framework Program (contract FP7-HEALTH-2011–282562).

PLCO was supported by the Intramural Research Program of the Division of Cancer Epidemiology and Genetics and by contracts from the Division of Cancer Prevention, National Cancer Institute, NIH, DHHS.

REFERENCES

1. Hocking JS, Stein A, Conway EL, Regan D, Grulich A, Law M, et al. Head and neck cancer in Australia between 1982 and 2005 show increasing incidence of potentially HPV-associated oropharyngeal cancers. British journal of cancer. 2011;104:886-91.

2. Blomberg M, Nielsen A, Munk C, Kjaer SK. Trends in head and neck cancer incidence in Denmark, 1978-2007: focus on human papillomavirus associated sites. International journal of cancer Journal international du cancer. 2011;129:733-41.

3. Reddy VM, Cundall-Curry D, Bridger MW. Trends in the incidence rates of tonsil and base of tongue cancer in England, 1985-2006. Annals of the Royal College of Surgeons of England. 2010;92:655-9.

4. Syrjanen S. HPV infections and tonsillar carcinoma. Journal of clinical pathology. 2004;57:449-55.

5. Ioka A, Tsukuma H, Ajiki W, Oshima A. Trends in head and neck cancer incidence in Japan during 1965-1999. Japanese journal of clinical oncology. 2005;35:45-7.

6. Braakhuis BJ, Visser O, Leemans CR. Oral and oropharyngeal cancer in The Netherlands between 1989 and 2006: Increasing incidence, but not in young adults. Oral oncology. 2009;45:e85-9.

7. Chaturvedi AK, Engels EA, Anderson WF, Gillison ML. Incidence trends for human papillomavirus-related and -unrelated oral squamous cell carcinomas in the United States. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2008;26:612-9.

8. Gillison ML, Alemany L, Snijders PJ, Chaturvedi A, Steinberg BM, Schwartz S, et al. Human papillomavirus and diseases of the upper airway: head and neck cancer and respiratory papillomatosis. Vaccine. 2012;30 Suppl 5:F34-54.

9. Chaturvedi AK, Anderson WF, Lortet-Tieulent J, Curado MP, Ferlay J, Franceschi S, et al. Worldwide trends in incidence rates for oral cavity and oropharyngeal cancers. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2013;31:4550-9.

10. Chaturvedi AK, Engels EA, Pfeiffer RM, Hernandez BY, Xiao W, Kim E, et al. Human papillomavirus and rising oropharyngeal cancer incidence in the United States. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2011;29:4294-301.

11. Kreimer AR, Clifford GM, Boyle P, Franceschi S. Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2005;14:467-75.

12. D'Souza G, Kreimer AR, Viscidi R, Pawlita M, Fakhry C, Koch WM, et al. Case-control study of human papillomavirus and oropharyngeal cancer. The New England journal of medicine. 2007;356:1944-56.

13. Nasman A, Attner P, Hammarstedt L, Du J, Eriksson M, Giraud G, et al. Incidence of human papillomavirus (HPV) positive tonsillar carcinoma in Stockholm, Sweden: an epidemic of viral-induced carcinoma? International journal of cancer Journal international du cancer. 2009;125:362-6.

14. Hong AM, Grulich AE, Jones D, Lee CS, Garland SM, Dobbins TA, et al. Squamous cell carcinoma of the oropharynx in Australian males induced by human papillomavirus vaccine targets. Vaccine. 2010;28:3269-72.

15. Kreimer AR, Chaturvedi AK. HPV-associated Oropharyngeal Cancers--Are They Preventable? Cancer Prev Res (Phila). 2011;4:1346-9.

16. Herrero R, Castellsague X, Pawlita M, Lissowska J, Kee F, Balaram P, et al. Human papillomavirus and oral cancer: the International Agency for Research on Cancer multicenter study. Journal of the National Cancer Institute. 2003;95:1772-83.

17. Zumbach K, Hoffmann M, Kahn T, Bosch F, Gottschlich S, Gorogh T, et al. Antibodies against oncoproteins E6 and E7 of human papillomavirus types 16 and 18 in patients with head-and-neck squamous-cell carcinoma. International journal of cancer Journal international du cancer. 2000;85:815-8.

18. Smith EM, Ritchie JM, Pawlita M, Rubenstein LM, Haugen TH, Turek LP, et al. Human papillomavirus seropositivity and risks of head and neck cancer. International journal of cancer Journal international du cancer. 2007;120:825-32.

19. Dahlstrom KR, Adler-Storthz K, Etzel CJ, Liu Z, Dillon L, El-Naggar AK, et al. Human papillomavirus type 16 infection and squamous cell carcinoma of the head and neck in never-smokers: a matched pair analysis. Clinical cancer research : an official journal of the American Association for Cancer Research. 2003;9:2620-6.

20. Furniss CS, McClean MD, Smith JF, Bryan J, Nelson HH, Peters ES, et al. Human papillomavirus 16 and head and neck squamous cell carcinoma. International journal of cancer Journal international du cancer. 2007;120:2386-92.

21. Pintos J, Black MJ, Sadeghi N, Ghadirian P, Zeitouni AG, Viscidi RP, et al. Human papillomavirus infection and oral cancer: a case-control study in Montreal, Canada. Oral oncology. 2008;44:242-50.

22. Applebaum KM, Furniss CS, Zeka A, Posner MR, Smith JF, Bryan J, et al. Lack of association of alcohol and tobacco with HPV16-associated head and neck cancer. Journal of the National Cancer Institute. 2007;99:1801-10.

23. Anantharaman D, Gheit T, Waterboer T, Abedi-Ardekani B, Carreira C, McKay-Chopin S, et al. Human papillomavirus infections and upper aero-digestive tract cancers: the ARCAGE study. Journal of the National Cancer Institute. 2013;105:536-45.

24. Ribeiro KB, Levi JE, Pawlita M, Koifman S, Matos E, Eluf-Neto J, et al. Low human papillomavirus prevalence in head and neck cancer: results from two large case-control studies in high-incidence regions. International journal of epidemiology. 2011;40:489-502.

25. Kreimer AR, Johansson M, Waterboer T, Kaaks R, Chang-Claude J, Drogen D, et al. Evaluation of human papillomavirus antibodies and risk of subsequent head and neck cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2013;31:2708-15.

26. Kreimer A, Brennan P, Lang Kuhs K, Waterboer T, Clifford G, Franceschi S, et al. Human papillomavirus antibodies and future risk of anogenital cancer: an nested case-control study in the European Prospective Investigation into Cancer and Nutrition (EPIC) study. Submitted. 2014.

27. Kreimer AR JM, Lang Kuhs KA, Waterboer T, Hildesheim A, Katki H, Chaturvedi A, Purdue M, Yu K, Pinsky P, Zhu C, Pawlita M, Brennan P. Validation of human papillomavirus type 16 E6 antibodies as a marker for oropharyngeal cancer: analysis in a US cohort. In Process.

28. Riboli E, Hunt KJ, Slimani N, Ferrari P, Norat T, Fahey M, et al. European Prospective Investigation into Cancer and Nutrition (EPIC): study populations and data collection. Public health nutrition. 2002;5:1113-24.

29. Prorok PC, Andriole GL, Bresalier RS, Buys SS, Chia D, Crawford ED, et al. Design of the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial. Controlled clinical trials. 2000;21:273S-309S.

30. Lagiou P, Georgila C, Minaki P, Ahrens W, Pohlabeln H, Benhamou S, et al. Alcohol-related cancers and genetic susceptibility in Europe: the ARCAGE project: study samples and data collection. Eur J Cancer Prev. 2009;18:76-84.

31. Waterboer T, Sehr P, Pawlita M. Suppression of non-specific binding in serological Luminex assays. Journal of immunological methods. 2006;309:200-4.

32. Waterboer T, Sehr P, Michael KM, Franceschi S, Nieland JD, Joos TO, et al. Multiplex human papillomavirus serology based on in situ-purified glutathione s-transferase fusion proteins. Clinical chemistry. 2005;51:1845-53.

33. Sitas F, Egger S, Urban MI, Taylor PR, Abnet CC, Boffetta P, et al. InterSCOPE study: Associations between esophageal squamous cell carcinoma and human papillomavirus serological markers. Journal of the National Cancer Institute. 2012;104:147-58.

34. Waterboer T, Dondog B, Michael KM, Michel A, Schmitt M, Vaccarella S, et al. Dried blood spot samples for seroepidemiology of infections with human papillomaviruses, Helicobacter pylori, Hepatitis C Virus, and JC Virus. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology. 2012;21:287-93.

35. Combes JD, Pawlita M, Waterboer T, Hammouda D, Rajkumar T, Vanhems P, et al. Antibodies against high-risk human papillomavirus proteins as markers for invasive cervical cancer. International journal of cancer Journal international du cancer. 2014;135:2453-61.

36. Holzinger D BL, Wichmann G, Michel A, Hofler D, Schmitt M, Boscolo-Rizzo P, Herold-Mende C, Boehm A, Del Mistro A, Bosch FX, Dietz A, Dyckhoff G, Pawlita M. Sensitivity of antibodies against HPV16 E6 and other early proteins for detection of HPV16-driven head and neck cancer. 29th International Human Papillomavirus Conference, Seattle Washington, August 21-25 2014.

Table 1: Participant characteristics overall and by study

	All Studies		**ARCAGE**		**EPIC**		**PLCO**	
Characteristics	N= 4666		N= 1425		N= 2317		N= 924	
	N	%	N	%	N	%	N	%
Gender								
Male	2972	63.7%	1059	74.3%	1165	50.3%	748	81.0%
Female	1694	36.3%	366	25.7%	1152	49.7%	176	19.0%
Age								
≤60	2782	59.6%	742	52.1%	1726	74.5%	314	34.0%
61-70	1436	30.8%	417	29.3%	504	21.8%	515	55.7%
>70	448	9.6%	266	18.7%	87	3.8%	95	10.3%
Region of Origin[1]								
Eastern Europe	185	4.0%	185	13.0%	0	0.0%	0	0.0%
Northern Europe	1430	30.6%	296	20.8%	1134	48.9%	0	0.0%
Southern Europe	1324	28.4%	757	53.1%	567	24.5%	0	0.0%
Western Europe	803	17.2%	187	13.1%	616	26.6%	0	0.0%
United States	924	19.8%	0	0.0%	0	0.0%	924	100.0%
Smoking[2]								
Never	1770	37.9%	516	36.2%	1040	44.9%	214	23.2%
Former	1614	34.6%	506	35.5%	718	31.0%	390	42.2%
Current	1242	26.6%	403	28.3%	519	22.4%	320	34.6%
Alcohol Consumption[2]								
Never	475	10.2%	172	12.1%	148	6.4%	155	16.8%
Ever	3745	80.3%	1252	87.9%	1782	76.9%	711	76.9%

Abbreviations: European Prospective Investigation Into Cancer and Nutrition (EPIC); Alcohol-Related Cancers and Genetic Susceptibility in Europe (ARCAGE); Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial

(PLCO); mean fluorescence intensity (MFI)
[1]Northern Europe includes: Denmark, Great Britain, Ireland, Norway and Sweden
Southern Europe includes: Croatia, Greece, Italy and Spain
Western Europe includes: France, Germany and The Netherlands
Eastern Europe includes: Czech Republic/Slovakia
[2]Columns do not add to total due to missing data

Table 2: Univariate Analysis of Demographic Characteristics and HPV16 E6 Seropositivity Among Controls

		HPV16 E6 Seropositive			
	Total	Moderate		High	
Characteristics	N= 4666	N= 17	OR (95% CI)	N= 15	OR (95% CI)
	N	N (%)		N (%)	
Study					
All	4666	17 (0.4)	-	15 (0.3)	-
ARCAGE	1425	5 (0.4)	-	6 (0.4)	-
EPIC	2317	9 (0.4)	-	4 (0.2)	-
PLCO	924	3 (0.3)	-	5 (0.5)	-
Gender					
Male	2972	11 (0.4)	Ref	10 (0.3)	Ref
Female	1694	6 (0.4)	1.0 (0.3-2.8)	5 (0.3)	0.9 (0.2-2.8)
Age					
≤60	2782	9 (0.3)	Ref	9 (0.3)	Ref
61-70	1436	6 (0.4)	1.3 (0.4-4.1)	5 (0.3)	1.1 (0.3-3.6)
>70	448	2 (0.4)	1.4 (0.1-6.7)	1 (0.2)	0.7 (0.0-5.0)
Region of Origin[1]					
Northern Europe	1430	3 (0.2)	Ref	4 (0.3)	Ref
Southern Europe	1324	6 (0.5)	2.2 (0.5-13.4)	3 (0.2)	0.8 (0.1-4.8)
Eastern Europe	185	1 (0.5)	2.6 (0.0-32.3)	0 (0.0)	-
Western Europe	803	4 (0.5)	2.4 (0.4-16.3)	3 (0.4)	1.3 (0.2-7.9)
United States	924	3 (0.3)	1.6 (0.2-11.6)	5 (0.5)	1.9 (0.4-9.8)

Smoking[2]					
Never	1770	9 (0.5)	Ref	2 (0.1)	Ref
Former	1614	4 (0.2)	0.5 (0.1-1.8)	10 (0.6)	5.5 (1.2-51.8)
Current	1242	4 (0.3)	0.6 (0.1-2.3)	3 (0.2)	2.1 (0.2-25.7)
Alcohol Consumption[2]					
Never	475	2 (0.4)	1.0 (0.1-4.5)	0 (0.0)	N/E
Ever	3745	15 (0.4)	Ref	12 (0.3)	Ref

Abbreviations: European Prospective Investigation Into Cancer and Nutrition (EPIC); Alcohol-Related Cancers and Genetic Susceptibility in Europe (ARCAGE); Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO); Not Estimateable (N/E)
[1]Northern Europe includes: Denmark, Great Britain, Ireland, Norway and Sweden
Southern Europe includes: Croatia, Greece, Italy and Spain
Western Europe include: France, Germany and The Netherlands
Eastern Europe includes: Czech Republic/Slovakia
[2]Columns do not add to total due to missing data

Table 3: Association of HPV16 L1, E1, E2, E4 and E7 Seropositivity with Moderate and High HPV16 E6 Seroreactivity Among Controls

	HPV16 E6 Seronegative			**HPV16 E6 Seropositive**					
				Moderate			High		
HPV16 Proteins	Total	No. Positive (%)	OR	Total	No. Positive (%)	OR (95%CI)	Total	No. Positive (%)	OR (95%CI)
Any[1]	4634	1245 (27.9)	Ref	17	5 (29.4)	1.1 (0.3-3.5)	15	7 (46.7)	2.4 (0.7-7.5)
HPV16 L1	4634	484 (10.4)	Ref	17	1 (5.9)	0.5 (0.0-3.5)	15	5 (33.3)	4.3 (1.1-13.8)
HPV16 E1	4632	155 (3.3)	Ref	17	0 (0.0)	-	15	1 (6.7)	2.1 (0.0-13.7)
HPV16 E2	4634	145 (3.1)	Ref	17	1 (5.9)	1.9 (0.0-12.6)	15	3 (20.0)	7.7 (1.4-29.1)
HPV16 E4[2]	3718	367 (9.9)	Ref	14	3 (21.4)	2.5 (0.4-9.5)	10	2 (20.0)	2.3 (0.2-11.5)
HPV16 E7	4634	345 (7.4)	Ref	17	1 (5.9)	0.8 (0.0-5.0)	15	1 (6.7)	0.9 (0.0-5.9)
Positive for any 2[1]	4634	221 (4.8)	Ref	17	1 (5.9)	1.2 (0.0-8.1)	15	2 (13.3)	3.1 (0.3-13.7)
Positive for any 3[1]	4634	28 (0.6)	Ref	17	0 (0.0)	-	15	2 (13.3)	25.3 (2.6-119.6)
Positive for any 4[1]	4634	2 (0.0)	Ref	17	0 (0.0)	-	15	0 (0.0)	-
Positive for any 5[1]	4634	0 (0.0)	Ref	17	0 (0.0)	-	15	0 (0.0)	-

[1]In addition to HPV16 E6

[2]Seroreactivity against HPV16 E4 was not assessed in PLCO

Table 4: Association of non-HPV16 Type E6 Seropositivity with Moderate and High HPV16 E6 Seroreactivity Among Controls

	HPV16 E6 Seronegative			**HPV16 E6 Seropositive**					
				Moderate			High		
HPV E6 Proteins	Total	No. Positive (%)	OR	Total	No. Positive (%)	OR (95% CI)	Total	No. Positive (%)	OR (95% CI)
Any	4634	368 (7.9)	Ref	17	4 (23.5)	3.6 (0.8-11.6)	15	3 (20.0)	2.9 (0.5-10.8)
HPV6	4634	26 (0.6)	Ref	17	1 (5.9)	11.1 (0.3-76.6)	15	0	-
HPV11	4634	90 (1.9)	Ref	17	0	-	15	1 (6.7)	3.6 (0.1-24.2)
HPV18	4634	56 (1.2)	Ref	17	1 (5.9)	5.1 (0.1-34.0)	15	0	-
HPV31	4634	85 (1.8)	Ref	17	1 (5.9)	3.3 (0.1-22.0)	15	0	-
HPV33	4634	40 (0.9)	Ref	17	0	-	15	2 (13.3)	17.7 (1.9-81.8)
HPV45	4634	65 (1.4)	Ref	17	0	-	15	0	-
HPV52[1]	3920	48 (1.2)	Ref	14	1 (7.1)	6.2 (0.1-42.9)	14	0	-

[1]Seroreactivity against the E6 protein of HPV52 was not determined in the EPIC anogenital study

Supplemental Table 1: Study-specific definitions for categorization of tobacco and alcohol use.

	Smoking			**Alcohol Use**	
Study	Current	Never	Former	Ever	Never
EPIC	Individuals who ever reported smoking a cigarette, pipe or cigar in their lifetime	Individuals who never smoked a cigarette, pipe or cigar in their lifetime	Individuals who had smoked in the past, but reported not smoking currently	Individuals who have ever consumed an alcoholic beverage	Individuals who have never consumed an alcoholic beverage
PLCO	Individuals who ever smoked cigarettes regularly for 6 months or longer	Individuals who never smoked a cigarette, pipe or cigar in their lifetime	Individuals who smoked in the past, but reported not smoking currently	Individuals who have ever consumed an alcoholic beverage	Individuals who have never consumed an alcoholic beverage
ARCAGE	Individuals who reported smoking any tobacco product at least once a week for a year	Individuals who reported never using tobacco in their lifetime or smoking less than once a week for a year	Individuals who reported having quit smoking for at least 12 months	Individuals who ever reported consuming any alcoholic beverage	Individuals who had never consumed any alcoholic beverages in their lifetime or who had not consumed alcohol within the past 12 months

Abbreviations: European Prospective Investigation Into Cancer and Nutrition (EPIC); Alcohol-Related Cancers and Genetic Susceptibility in Europe (ARCAGE); Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO)